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Research Article

Citrus pectin oligomers in combination with cisplatin enhances apoptosis in HepG2 human hepatocellular carcinoma cells

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Abstract
Pectin is a widely used gelling agent in food, primarily composed of polygalacturonic acid. Cisplatin (DDP), a commonly used chemotherapeutic agent, has significant side effects, which necessitate efforts to reduce its usage. One approach to mitigate these side effects involves combining cisplatin with food-based bioactive compounds, such as oligogalacturonides (OGA) - shorter chain derivatives of pectin. In the present study, synergistic suppressive effects of OGA and DDP on hepatocellular carcinoma (HCC) cells were determined. HepG2 cells were treated with OGA (100 $\mu g.ml^{-1}$) and DDP (2 $\mu g.ml^{-1}$) in various combinations. Assays for cell viability, morphology, lactate dehydrogenase activity, cell cycle, apoptosis, western blot, galectin-3, and comet analysis were performed. Compared to DDP treatment, 55% reduction of IC_{50} values in the cell viability of HepG2 cells treated with DDP 12h + OGA 12h was observed. OGA-DDP combined showed cell cycle arrest in G2/M, increased galectin-3 release, and decreased mitochondrial membrane permeability (MMP) significantly ($p<0.05$). Western blot study showed the upregulation of Bax, p53, and Caspase-3, and the downregulation of Bcl-2 proteins ($p<0.05$) by OGA and DDP combination treatment. These findings suggest that OGA-DDP enhances cisplatin's anticancer efficacy while potentially reducing its required dosage, offering a promising strategy for improving HCC treatment outcomes.

Keywords
cisplatin, hepatocellular carcinoma cells, oligogalacturonide, synergistic effects, bioactive compounds

Abbreviations
HCC – Hepatocellular carcinoma; HepG2 – Hepatocellular carcinoma; DNA – Deoxyribonucleic acid; PBS – Phosphate-buffered saline; OGA – Oligogalacturonides; EDTA – Ethylenediaminetetraacetic acid; MMP – Mitochondrial membrane potential; LDH – Lactate dehydrogenase; TBE – Tris/Borate/EDTA; LM – Low melting; DAPI – 4',6-diamidino-2-phenylindole; Anexin V-FITC – Annexin V conjugated with fluorescein isothiocyanate

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Introduction

Liver cancer is the fourth leading cause of cancer death worldwide, accounting for over 800,000 deaths annually. Hepatocellular carcinoma (HCC) accounts for approximately 90% of primary liver cancers (Tan et al. 2024). Early-stage diagnosis was associated with more than 70% five-year survival rates, while advanced diagnosis reduces the survival rate to less than 20% (Calderon-Martinez et al. 2023). HCC is known to rapidly spread, metastasize, and frequently recur (Yang et al. 2015). HCC treatment is challenging because symptoms typically appear at an advanced stage, and routine early surveillance is uncommon, even in countries with sufficient medical resources (Yang et al. 2019). Another explanation is that the morbidity and mortality rates of HCC are similar as most HCC patients are often detected at a later stage when it is too late for successful treatment (Venook et al. 2010).

Currently, there are no completely effective treatments (drug and non-drug therapies) for HCC, because all anticancer treatments have limitations, and the success of treatment greatly depends on the grade of tumor and stage of the disease (Chen et al. 2020). Although several drugs have been recently licensed in some countries, the success rate has been still poor (Bruix et al. 2021). Multiple treatment options exist for HCC. However, chemotherapy remains essential for advanced cases, especially when targeted therapy or immunotherapy is constrained by factors such as drug resistance, high costs, limited patient eligibility, or unpredictable responses (Ikeda et al. 2018). Targeted therapy is restricted to specific genetic profiles and prone to resistance (Shi et al. 2023), while immunotherapy, though effective in some cases, has variable response rates and immune-related adverse effects (Yin et al. 2023). Nevertheless, it is well-documented that chemotherapy can have cytotoxic effects on normal cells and also may induce secondary malignancies in the long run (Bennukul et al. 2014). Various signalling pathways are altered in HCC that are closely associated with HCC and several genetic pathways have been identified involving disruption of p53, PIK3CA, and β-catenin pathways (Waller et al. 2015). The human hepatocellular carcinoma (HepG2) cell line is a hepatoblastoma cell line commonly used to study the pathogenesis and therapy of HCC as it provides sustainable proliferation, stable phenotype, easy accessibility, and manipulation (Gerets et al. 2012).

Cisplatin (DDP), a platinum-based chemotherapy is one of the most effective anticancer chemotherapeutics and is commonly used to treat various types of cancers including HCC (Tchounwou et al. 2021). It has been shown to have cytotoxic properties and successfully limit the spread of HCC by disrupting the cell cycle (Wang et al. 2015). In addition, it also targets the DNA, RNA, and proteins that have an affinity to the nucleus, inhibits DNA replication, and damages the DNA (Galluzzi et al. 2012). Once formed, this complex triggers a cascade of cellular reactions that result in apoptosis and induce the recovery of damaged DNA. When the damage is extreme, apoptosis malfunction can result in loss of function in regulatory pathways, thus, increasing the chance of tumor formation (Olsson and Zhivotovsky, 2011). Cisplatin is administered by intra-arterial route to treat HCC (Hamaya et al. 2023). It is generally administered through the hepatic artery with a total dose of 65 $mg.m^{-2}$, which is adjusted according to the patient’s condition such as renal function and platelet counts (Osaki et al. 2013). Cisplatin has a narrow therapeutic window and has also been shown to develop drug resistance by decreasing intracellular accumulation of cisplatin, increasing detoxification, increasing DNA repair, and preventing apoptosis (Hamaya et al. 2023). Increasing the therapeutic can lead to other side effects. When treated solely with cisplatin, some individuals experienced severe side effects and others have shown significant resistance to the drug (Tchounwou et al. 2021). These mechanisms of DDP include (1) decreased intracellular accumulation of DDP, (2) increased detoxification through increased intracellular thiol levels (3) increased DNA repairing activity, and (4) prevention of apoptosis through signalling molecules (Hamaya et al. 2023). Therefore, to improve chemotherapy efficiency and overcome resistance, cisplatin is commonly used in combination with other drugs.

Pectin is a natural compound found in fruits and vegetables and is a component of cell walls in all higher plants. Pectin-derived oligomers also known as oligogalacturonides (OGA) made from hydrolysis reaction have demonstrated antiproliferative activity against erythroleukemia

K562 cells as well as colon cancer HuCC and HT-29 cells (Bergman et al. 2010). Their growth-inhibitory effects are linked to mechanisms such as lactate dehydrogenase activity modulation and galectin-3 release regulation in human cancer cells (Mukhopadhyay et al. 2013). Furthermore, OGA and cisplatin when used together showed a synergistic effect on human lung cancer as well as reduced kidney toxicity of cisplatin and delayed cisplatin resistance (Huang et al. 2018). Low molecular citrus pectin was shown to inhibit liver cancer cells (Wu et al. 2022). OGA shows promising anti-cancer activity to reduce the usage of commercial medicine, however, the information on the efficacy of cisplatin and OGA combination and sequence on HepG2 cell lines is lacking. Therefore, in the present study, we investigated the suppressive effect of citrus OGA and cisplatin combination against HCC.

Materials and Methods

The pectin enzyme used in the present study was Peclyve CP (Lallemand Australia Pty. Ltd., North Adelaide, Australia). The pectin enzyme contained 133.5 $U.ml^{-1}$ pectin lyase (PL) activity, 50.6 $U.ml^{-1}$ pectin methylase esterase (PME) activity, and 22.4 $U.ml^{-1}$ polygalacturonase (PG) activity isolated from *Aspergillus niger*. Citrus pectin (Nacalai Tesque, Kyoto, Japan) used in the present study had a 60% degree of esterification. Other chemicals used in this study were analytical grade.

Sample preparation of oligogalacturonide. Oligogalacturonide (OGA) was made by incubating citrus pectin and pectic enzyme at pH 4 and 45℃ for 48 h following an earlier study (Huang et al. 2018). In brief, 100 mL citrus pectin (1% *w/v*) with 5 mL commercial microbial pectic enzyme was incubated at pH 4, 45℃ for 48 h. Following incubation, OGA was boiled for 10 min and freeze-dried for future study. The resulting OGA had a molecular weight of less than 1 kDa with the degree of esterification less than 11.6 (Huang et al. 2012).

Cell cultures and treatments. The human hepatocellular carcinoma (HepG2) cell line used in the present study was purchased from Bioresource Collection and Research Centre in Hsinchu, Taiwan, and cultured in DMEM media containing 10% (v/v) FBS. The treatments on the cells were divided into (1) untreated HepG2 cells or control, (2) OGA 100 $\mu g.mL^{-1}$ for 24 h, (3) DDP 2 $\mu g.ml^{-1}$ for 24 h, (4) DDP 2 $\mu g.ml^{-1}$ combined with OGA 100 $\mu g.mL^{-1}$ for 24 h, (5) DDP 2 $\mu g.ml^{-1}$ for 12 h followed by OGA 100 $\mu g.mL^{-1}$ for 12 h, (6) OGA 100 $\mu g.mL^{-1}$ for 12 h followed by DDP 2 $\mu g/ml^{-1}$ for 12 h (Table 1).

Table 1. *In vitro* treatment in the study

Sample	First treatment (12h)	Second treatment (12h)
Control	None	None
OGA 24h	OGA	OGA
DDP 24h	DDP	DDP
DDP+OGA 24h	OGA+DDP	OGA+DDP
DDP 12h+OGA 12h	DDP	OGA
OGA 12h+DDP12h	OGA	DDP

*OGA represent oligogalacturonide 100 $\mu g.mL^{-1}$ and DDP represent cisplatin 2 $\mu g.mL^{-1}$.

Cell viability, morphology, lactate dehydrogenase, and western blot. Cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay using Microplate Spectrophotometer (BIO-TEK®, Vermont) for a 96-well plate with an initial cell density of 1×10^4 cells/well. Cell morphology was observed using a microscope (Primovert, Zeiss, Gottingen, Germany) and a fluorescence microscope (TissueGnostics, Wien, Austria) in a 6-well plate with an initial cell density of $1x10^5$ cells/well. For Lactate Dehydrogenase (LDH) Activity, a 96-well plate (1×10^5 cells/well) was measured following kit instructions (Sigma-Aldrich, MO). The protein quantity used in the Western blot analysis (60 μg) was measured using the Bradford method. The results were then analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) to calculate the relative ratio normalized to β-actin as the loading control. Detailed information about these methodologies can be found in Huang et al. (2018).

Cell cycle and apoptosis analyses. Cell cycle and apoptosis detection were conducted using 6-well plates at 1×10^5 cells/well. After treatment, HepG2 cells were harvested and fixed in 70% ethanol. After centrifugation, HepG2 cells were re-suspended in 1 mL PBS containing 10 $\mu g.mL^{-1}$ RNase A and 20 $\mu g.mL^{-1}$ propidium iodide (PI), following 30 min of incubation. For apoptosis detection, HepG2 cells were digested with trypsin and then washed three times with PBS. After centrifugation, HepG2 cells were re-suspended in 500 µL of binding buffer, and then 5 µL of FITC-conjugated Annexin-V and 5 µL of PI were added and the solution is incubated for 15 min. For both analyses, determinations were conducted on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) with 10,000 cells counting/sample by WinMDI version 2.9 (Scripps Research Institute, La Jolla, CA).

Galactin-3 analysis. Galactin-3 analysis was conducted using Huang et al. (2012) method with some modifications. As much as 5×10^4 cells/ml was placed into a 96-well plate (200 µl) for incubation at 37℃ for 24 h. The cells that didn't adhere were washed with 100 µl PBS and the sample was mixed with the adhered cells for a further 24 h incubation. Media (50 µL) from each well was moved into the Galactin-3 plate, added with 50 µl sample diluent, and left for 1 h. Then, the plate was washed 4 times with wash buffer, added with 100 µL TMB substrate (3,3',5,5'-tetramethylbenzidine) solution, and incubated for 30 min. After incubation, a stop solution was added, and the absorbance was read at 450 nm wavelength.

Comet assay. The comet assay was performed following the method of Yang et al. (2015), with modifications in the treatment duration and sample used. Initially, cells at a concentration of 1×10^5 cells/mL were seeded into a 6-well plate (3 mL) and incubated at 37℃ for 24 h. Non-adherent cells were then removed by washing with 1 mL of PBS, after which the sample was combined with the adhered cells and incubated for an additional 24 hours. Cells were harvested using 0.5 ml trypsin-EDTA by 3-5 min exposure and centrifuged at 1000 rpm for 5 min. Harvested cells were then washed with PBS and diluted into 2.5 x 10^5 cells/ml. Cells were then mixed with LM agarose with a ratio of 1:10 and 50 µl of solution was added into Comet slide (2 samples/slide) and was allowed to solidify at 4℃ for 30 min. After removing the coverslip, the slides were dipped in the pre-chilled lysis buffer for 60 minutes at 4℃ and then denatured using TBE buffer for 15 min. Treated slides were then placed into electrophoresis in an electric field for 40 min at 4℃. After electrophoresis, the DNA was washed with double distilled water and 70% ethanol for 5 min each followed by DNA colouring using 5 µg.ml^{-1} propidium iodide for 30 min and observed under a fluorescence microscope.

Statistical analysis. All experiments were conducted with three replicates and three repetitions. Results are expressed as mean ± standard deviation and one-way ANOVA was used for comparing treatment groups, followed by Duncan's post-hoc using SPSS Version 21 (SPSS Inc., Chicago, IL, USA) at $p \leq 0.05$.

Results and Discussion

Effect of OGA and DDP on cell viability and LDH release. The increase in HepG2 cells was evaluated using the MTT assay after treating the cells with OGA and DDP (concentration 2-8 µg.mL^{-1}) for 24 h (Table 2). The result showed growth inhibition increased by 14.7% in OGA and 18-68% in DDP. The combined non-sequential treatment showed a synergistic effect with a 1.12-fold improvement of DDP towards HepG2 cells at a concentration of 6 µg.mL^{-1}. In combined sequential treatment, OGA 12 h + DDP 12 h displayed the highest synergistic effect with 2.71-fold at 2 µg.mL^{-1}. At the same concentration, DDP 12 h + OGA 12 h presented the second-highest synergistic effect with 2.68-fold. These results showed that OGA could enhance the synergistic effect of DDP with the best results at combined sequential treatment.

For further analysis, the concentration that inhibited 50% cell growth (IC_{50}) at 24 h cultivation was measured (Table 3 left). DDP 24 h treatment showed the highest IC_{50} which was 6.4 µg.mL^{-1}. Treatment with DDP 12 h + OGA 12 h presented the lowest IC_{50} while the difference in IC_{50} between DDP + OGA 24 h and OGA 12 h + DDP 12 h were not statistically significant.

Cell viability can also be assessed by measuring lactate dehydrogenase (LDH) released into the media, which occurs when the loss of cell membrane integrity leads to the leakage of the LDH enzyme from dead cells (Al-Qubaisi et al. 2013). The higher the LDH released indicated the higher number of dead cells. As shown in Table 3 (right), the OGA-DDP combination showed significantly higher LDH activity compared to OGA and DDP 24 h, individually. The combination of DDP for 12 h followed by OGA for 12 h showed the highest LDH activity with a 20.6% synergistic effect compared to the combination of individual 24-h treatments of

OGA and DDP. This suggests that DDP 12h + OGA 12h was more effective at inducing cytotoxicity in HepG2 cells than both the DDP + OGA 24h treatment and the OGA 12h + DDP 12 h sequence. These findings also revealed that the sequence influenced the cytotoxicity. Cells treated with DDP first showed higher cytotoxicity compared to cells treated with OGA, showing a sequence-based synergetic effect. It may suggest that OGA works better on the HepG2 cell has been weakened by DDP. Compared to the Annexin V results for apoptosis, the findings suggest that cell death occurs through both apoptotic and necrotic pathways.

Effect of OGA and DDP on morphological changes. An inverted light microscope examined the effect of the OGA-DDP combination in HepG2 cells (Fig. 1 left). DDP and OGA treatment showed severe changes in morphology, including cell shrinkage, the presence of apoptotic nuclear bodies, and a decrease in cell count attached to the plate. The combined OGA and DDP treatment showed lower cell numbers than DDP and OGA alone. Among the combinations, treatment with DDP 12h + OGA 12h showed the lowest cell number and highest apoptotic cells. Therefore, OGA-DDP combination treatment indicated an improved effect on the DNA of HepG2.

Fig. 1 (right) displays DAPI-stained HepG2 cells under a fluorescence microscope. The collected HepG2 cells revealed that the DDP 12h + OGA 12h treatment caused severe changes in the shape of the apoptotic nucleic body as indicated with a red arrow, while an example of a normal cell is indicated with a white arrow.

Table 2. Growth inhibition of DDP and OGA on human liver cancer HepG2 cells

Treatment	DDP, μg.mL^{-1}					
	0	2	4	6	8	10
OGA 24h	14.7±2.5	-	-	-	-	-
DDP 24h	-	18.2±2.9^{eC}	32.6±3.1^{dC}	48.5± 3.0^{cD}	57.7±2.2^{bC}	68.4±0.6^{aB}
DDP+OGA 24h	-	23.0±1.4^{dB}	48.5±0.8^{cB}	70.5±1.9^{bB}	71.3±0.3^{bB}	74.4±1.1^{aA}
DDP 12h+OGA 12h	-	45.1±1.6^{cA}	69.0±2.0^{bA}	76.5±1.7^{aA}	74.8±0.5^{aA}	76.5±0.3^{aA}
OGA 12h+DDP12h	-	45.6±1.6^{cA}	50.9±1.3^{bB}	57.4±2.8^{aC}	53.4±0.8^{bD}	56.6±1.2^{aC}

*Cells were cultivated with OGA, DDP, DDP+OGA, DDP then OGA, and OGA then DDP at 37℃ for a total of 24 h. Means expressed as mean ± SD of three measurements. Means bearing the same superscript capital letters in the same column, and the same superscript small letters in the same row are not significantly different ($p < 0.05$) analyzed by one-way ANOVA with Duncan's post hoc

Table 3. The IC_{50} value of cell viability and LDH released of OGA and DDP treatment on HepG2 cells

Treatment	IC_{50}, DDP μg.mL^{-1}	LDH released, %
Control	ND	0.0±3.5^{e}
OGA 24h	ND	30.8±1.1^{d}
DDP 24h	6.4±0.4^{a}	30.0±2.2^{d}
DDP+OGA 24h	4.0±0.0^{b}	53.3±2.8^{b}
DDP 12h+OGA 12h	2.9±0.4^{c}	73.3±2.8^{a}
OGA 12h+DDP12h	3.9±0.1^{b}	36.5±3.5^{c}

*Cells were cultivated with OGA, DDP, DDP+OGA, DDP then OGA, and OGA then DDP at 37℃ for 24 h. Data expressed as mean ± SD in each column with the same superscript small letters are not significantly different ($p < 0.05$) analyzed by one-way ANOVA with Duncan's post hoc. DDP: cisplatin at 2 μg.ml^{-1}, OGA: oligogalacturonides at 100 μg.ml^{-1}, ND: not determined

Effect of OGA and DDP on cell cycle regulation and annexin V indicated apoptosis. The phase of cell cycle arrest (Table 4) on HepG2 was determined by flow cytometry. Based on the cell cycle analysis, an increase in the Sub-G1 phase was observed, indicating apoptosis, which was further confirmed using Annexin V-FITC (Fig. 2). Additionally, a high percentage of cells in the G2/M phase suggests the possibility of cell cycle arrest at this stage. Based on Annexin V-FITC, DDP 24h showed that most cells endured early apoptosis whereas DDP + OGA 24h showed most cells endured late apoptosis.

Effect of OGA and DDP on Western blot analysis. The expression of apoptotic genes and proteins was examined to assess the apoptotic events in the cells. Western blot results showed that OGA 24h increased pro-caspase 3 significantly but did not show a significant effect on p 53 and cleaved-caspase 3 (Fig. 3). On the other hand, DDP 24 h significantly increased p53, pro-caspase 3, and cleaved-caspase 3. Both OGA and DDP 24 h didn't significantly affect pro-apoptosis Bax and anti-apoptosis Bcl-2. In the combined treatment, OGA significantly increased p53, pro-caspase 3, cleaved-caspase3, and pro-apoptosis Bax while decreasing anti-apoptosis Bcl-2.

Effect of OGA and DDP on galectin-3 and mitochondrial membrane potential (MMP). Results of galectin-3 and Mitochondrial Membrane Potential (MMP) of HepG2 cells after treatment with OGA and DDP are shown in Table 5. After 24 h culture, the amount of secreted galectin-3 in the HepG2 control group did not show a significant difference compared to the OGA and DDP 24 h group. The combined treatment significantly increased the concentration of galectin-3 in HepG2 with DDP 12h + OGA 12h showing the highest increase. The increase in galectin-3 at DDP + OGA 24h and OGA 12 h + DDP 12 h was not statistically different. It has been reported that disruption of mitochondrial membrane potential causes irreversible cell death and the release of cytochrome C (Tait and Green 2010).

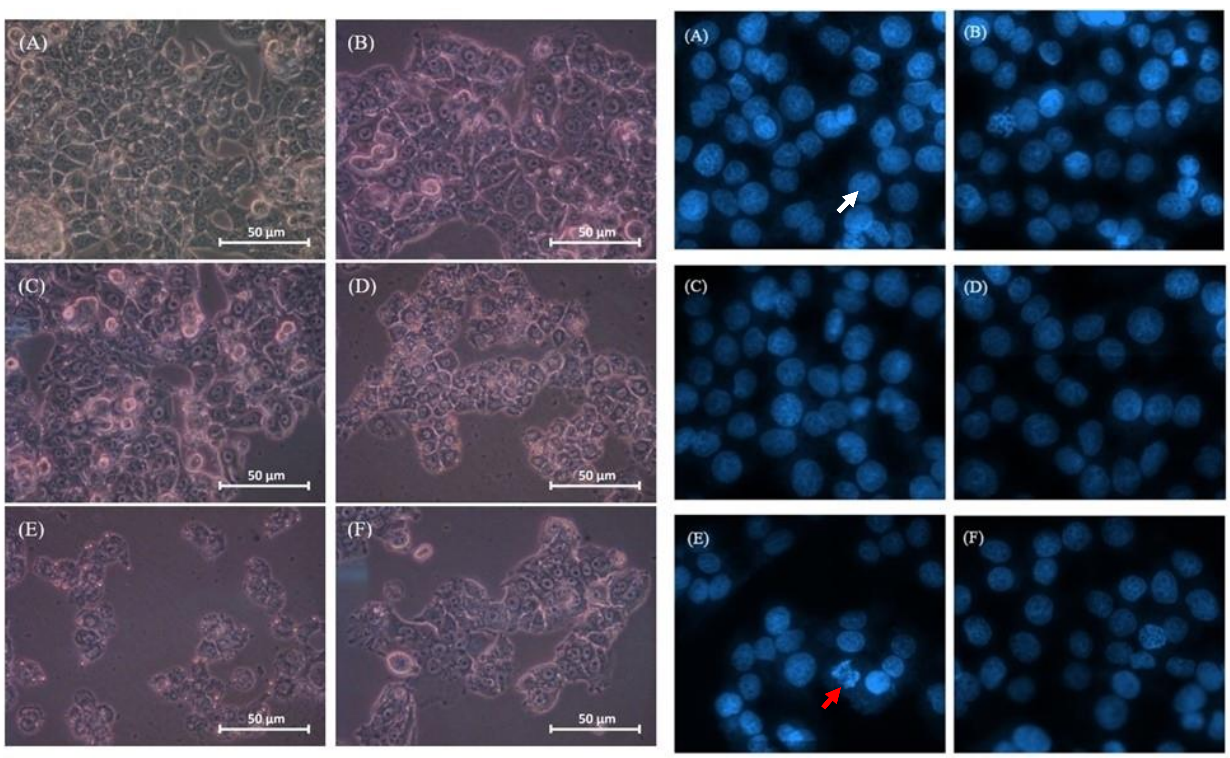

Figure 1. Morphology (left) and DAPI fluorescence staining (right) of DDP and OGA treated human liver cancer HepG2 cells. (A) Control, (B) OGA 24h, (C) DDP 24h, (D) DDP+OGA 24h, (E) DDP 12h+OGA 12h, (F) OGA 12h+DDP 12h. Control: untreated HepG2 cells, DDP: cisplatin (2 μg.ml^{-1}), OGA: oligogalacturonides (100 μg.ml^{-1}). White arrow shows normal cell and red arrow shows apoptotic cell

Table 4. Flow cytometry analysis of cell cycle in HepG2 cells after DDP and OGA treatment

Treatment	Cell population, %			
	Sub G1	**G1/G0**	**S**	**G2/M**
Control	1.2±0.1^{f}	37.4±1.4^{a}	11.4±0.2^{a}	50.1±1.5^{d}
OGA 24h	7.8±0.2^{e}	30.2±0.5^{b}	8.2±0.4^{b}	53.9±0.8^{c}
DDP 24h	10.4±0.5^{d}	18.6±0.2^{c}	6.2±0.3^{d}	64.9±0.4^{b}
DDP+OGA 24h	13.3±0.4^{b}	12.4±0.2^{d}	4.5±0.2^{e}	69.8±0.4^{a}
DDP 12h+OGA 12h	41.3±0.4^{a}	5.6±0.1^{e}	7.1±0.2^{c}	46.1±0.7^{e}
OGA 12h+DDP12h	12.4±0.1^{c}	29.5±0.2^{b}	7.4±0.1^{c}	50.7±0.1^{d}

*HepG2 cells were treated with 100 μg.mL^{-1} of OGA (oligogalacturonides) or 2 μg.mL^{-1} of DDP (cisplatin) at 37°C for 24 h. Data expressed as mean ± SD each column (cell cycle) with the same superscript small letters are not significantly different ($p < 0.05$). DDP: cisplatin at 2 μg.ml^{-1}, OGA: oligogalacturonides at 100 μg.ml^{-1}.

To further understand the mechanism of OGA-DDP-induced HepG2 cell death, we measured the mitochondrial membrane potential. The MMP significantly decreased when HepG2 cells were co-treated with OGA and DDP as compared with the control group (Table 5). Treatment with OGA 24 h showed the highest decrease in MMP while DDP 24 h showed the lowest decrease. This reduction in MMP, along with the increased Bax/Bcl-2 ratio and cleaved caspase-3 levels, suggests that cytochrome c release may have been triggered, activating the intrinsic apoptotic pathway.

Olive tail moment of OGA-DDP combination treatment on HepG2 cells. The breakage in DNA strands of HepG2 cells was measured by the comet assay. When observed by fluorescence microscope, cells treated with DDP and OGA for 24 h showed breakage in DNA strands. The cells treated with DDP for 24 h showed a significant increase in the olive tail moment (OTM), and combination treatment increased it to 2-5-fold in comparison with the control (Fig. 4). However, OGA 24 h treatment didn't show a significant difference compared with the control.

Chemotherapy using cisplatin has been used successfully to suppress cancer cells. Cisplatin, when used solely has been known to result in undesirable side effects and chemo-resistance due to its efficient cytotoxic actions. For this reason, OGA adjuvant therapy has been recommended. In the present study, we studied apoptosis caused by the synergistic effect of OGA and DDP on HepG2 cells and attempted to identify the probable pathway of melatonin action. The present study showed that the combination of OGA and DDP, especially the combined sequential treatment, showed a significant reduction in cell viability and high LDH activity compared to OGA and DDP alone. Moreover, OGA can exhibit cytotoxicity against tumor cells by altering the membrane permeability (Huang et al. 2012). Meanwhile, DDP was shown to damage deoxyribose which resulted in the formation of aldehydes including malonaldehyde. On the other hand, one of the antioxidant compounds, flavonoids, is toxic to cancer cells and complicates cancer therapy (Liskova et al. 2020). This explained the low value of IC_{50} in the combined treatment of HepG2.

The findings also indicated that the cell cycle was arrested at the G2/M phase. This type of arrest is a control mechanism that stops the cell cycle at the transition between the G2 phase and mitosis (M phase). G2/M cell cycle arrest is a crucial cellular response to severe DNA damage. If the damage is irreparable, the cell initiates apoptosis (programmed cell death) to prevent the propagation of damaged DNA by activating p 53.

An increase in galectin-3 was found after cultivation with OGA and DDP. OGA-DDP combination resulted in higher galectin-3. Some studies suggest that cells may actively secrete galectin-3 in response to apoptotic stimuli (Díaz-Alvarez and Ortega 2017).

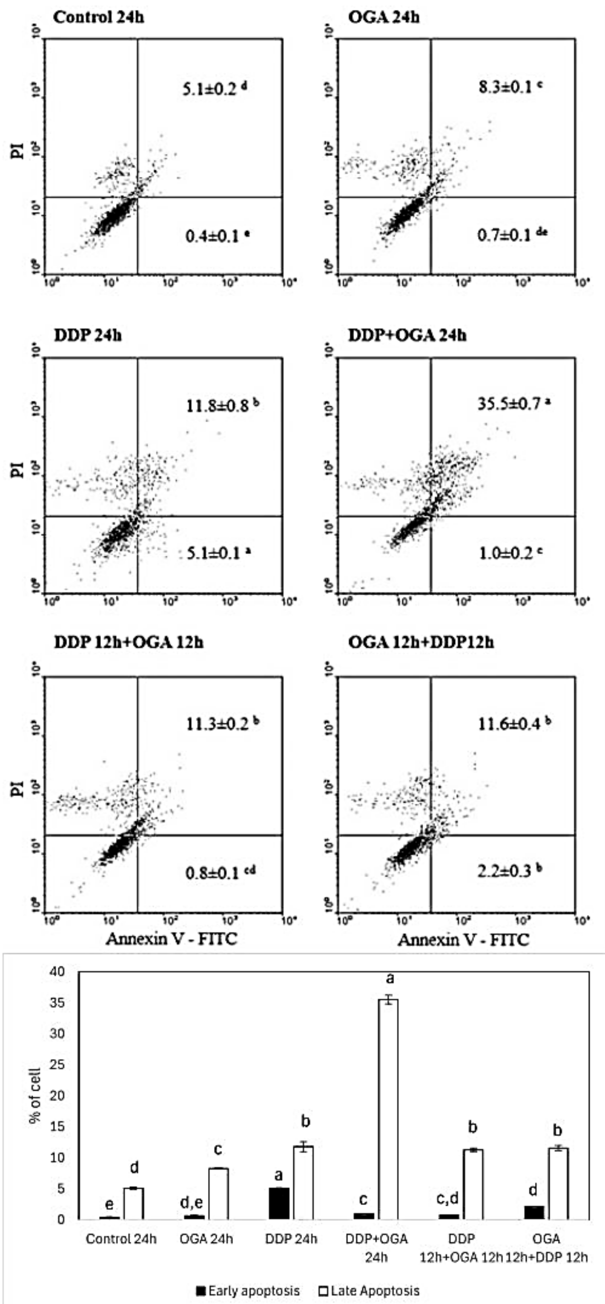

Figure 2. Flow cytometry analysis of apoptosis using Annexin V in HepG2 cells were treated with 100 µg.mL^{-1} of OGA (oligogalacturonides) or 2 µg.mL^{-1} of DDP (cisplatin) at 37°C for 24 h. Data expressed as mean ± SD with different superscript letters indicate statistical significance from three replications analyzed by one-way ANOVA with Duncan's post-hoc

Extended G2/M arrest may result in mitochondrial dysfunction, marked by a reduction in MMP (Cabrera et al. 2015), while OGA showed higher disruption in MMP compared to DDP on HepG2 cells. A reduction in MMP leads to the release of cytochrome C from mitochondria into the cytosol and apoptosome formation through cytochrome C and Apaf-1 binding which subsequently triggers the cascade of caspases, culminating in apoptosis (Alsherhri 2024). The increase in Bax expression and the decrease in Bcl-2 levels in combination treatments indicate MMP, while p53 upregulation may further enhance cytochrome C release.

The western blot result after DDP treatment showed an upregulation in p53, pro-caspase 3, and cleaved-caspase. There was an increase in phosphorylated p53, pro-caspase 3, cleaved-caspase 3, and Bax increased after OGA-DDP treatment in the HepG2 cell line, whereas anti-apoptotic Bcl-2 decreased. This demonstrates that OGA combined with DDP resulted in apoptosis in HepG2 via the intrinsic mitochondrial pathway by increasing p53 expression, which may relate to G2/M cycle arrest. Cisplatin demonstrated a pro-death effect, which is mainly regulated by p53 expression, which indicated that cisplatin affected the G2/M phase and is consistent with a previous study (Mueller et al. 2006). In the combined treatment, especially in DDP 12 h + OGA 12 h, the cell in the Sub G1 phases increases. Therefore, OGA could have increased the DDP effect on cell apoptosis through Sub G1 phases (Ding et al. 2010). Based on the findings from cell cycle arrest and Western blot analysis, we propose that the mechanism of action operates in a p53-dependent manner.

In this study, we used the alkaline comet assay for assessing the genotoxicity of DDP and OGA, on HepG2 cells. The alkaline comet assay is an important method for assessing DNA damage in a wide range of cell types (Mendonça et al. 2010) and its result expressed as OTM (Olive Tail Moment). Our result showed that the combination of DDP and OGA resulted in significantly higher OTM which represent higher damage to the DNA membrane compared to the control or DDP and OGA alone. This condition can result in LDH leakage and trigger apoptosis in cells (Ma et al. 2021).

The current findings in this research are limited to *in vitro* studies, which provide valuable insights, but do not fully replicate the complexity of living organisms. Therefore, further *in vivo* studies using

HepG2 xenograft models in mice could help validate the efficacy and safety of OGA-DDP combination therapy. Additionally, clinical investigations assessing pharmacokinetics, toxicity, and patient response would be necessary to explore its potential as a therapeutic approach for hepatocellular carcinoma.

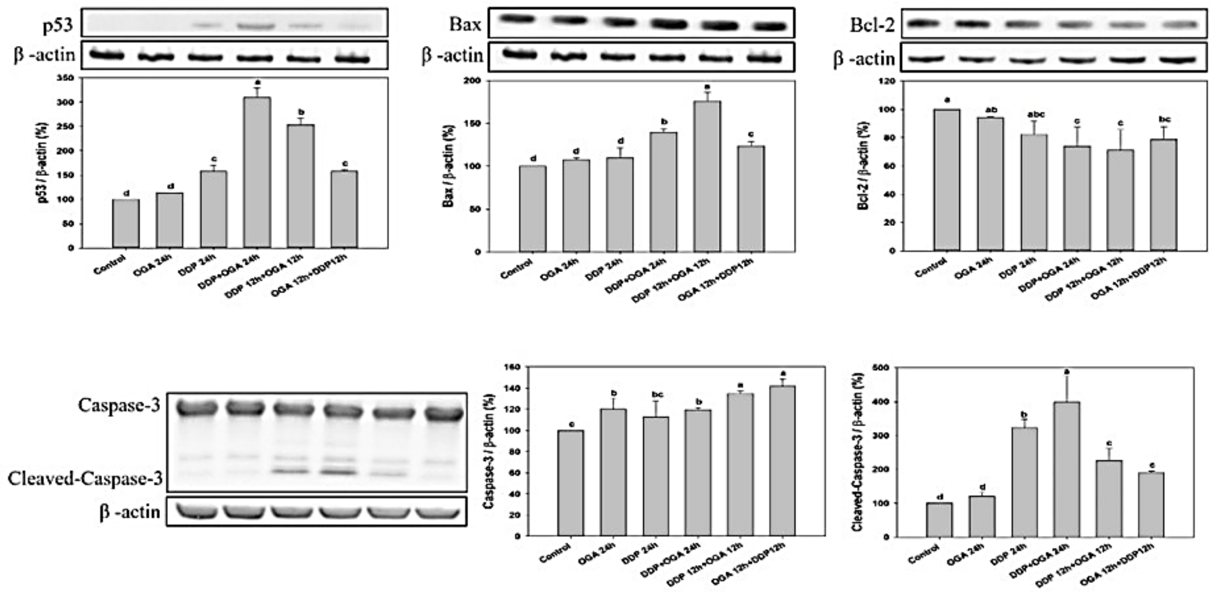

Figure 3. Densitometric analysis of western blot results for p53, Bax, Bcl-2, Caspase-3 and Cleaved-Caspase-3 normalized to β-actin and express as (%) with control as 100% or 1-fold. HepG2 cells were treated with 100 μg.mL^{-1} of OGA (oligogalacturonides) or 2 μg.mL^{-1} of DDP (cisplatin) at 37°C for 24 h. Data expressed as mean ± SD with different superscript letters indicate statistical significance from three replications analyzed by one-way ANOVA with Duncan's post-hoc

Table 5. Effects of OGA and DDP on galectin-3 and mitochondrial membrane potential of HepG2 cells

Treatment	Galectin-3, ng.mL^{-1}	Mitochondria membrane potential, %
Control	4.51±0.34^{c}	100.0±0.9^{a}
OGA 24h	5.19±0.35^{c}	51.6±0.8^{f}
DDP 24h	5.55±0.46^{c}	82.9±0.1^{b}
DDP+OGA 24h	12.17±0.91^{b}	77.9±1.6^{c}
DDP 12h+OGA 12h	14.18±1.26^{a}	72.5±0.4^{d}
OGA 12h+DDP12h	11.90±1.68^{b}	59.0±0.3^{e}

*HepG2 cells were treated with 100 μg.mL^{-1} of OGA (oligogalacturonides) or 2 μg.mL^{-1} of DDP (cisplatin) at 37°C for 24 h. Data expressed as mean ± SD with different superscript letters indicate statistical significance from three replications analyzed by one-way ANOVA with Duncan's post-hoc.

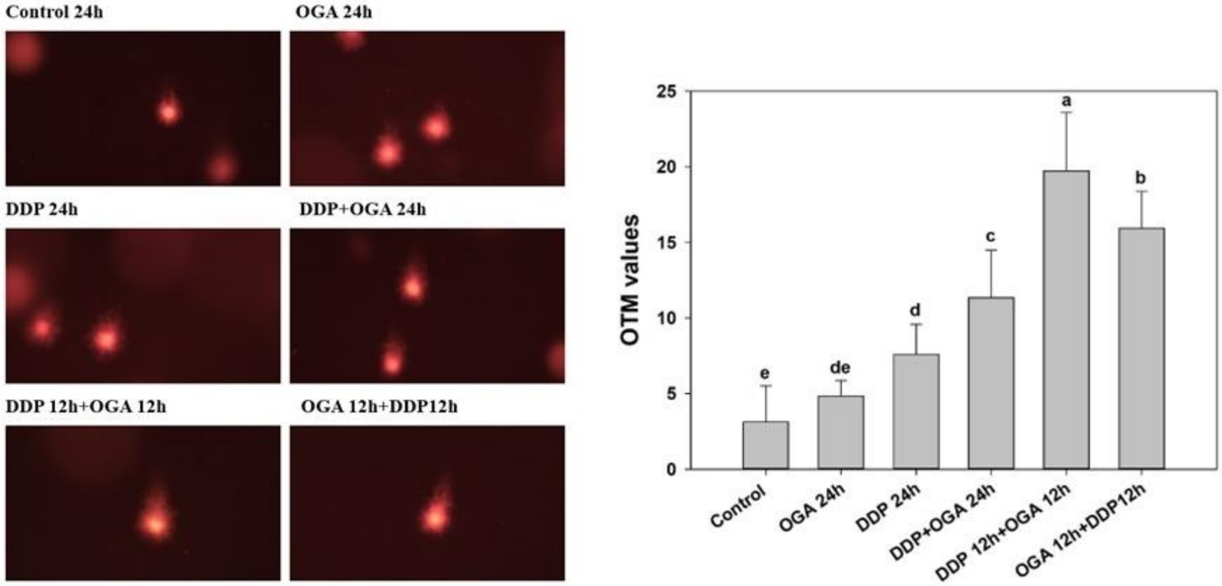

Figure 4. Comet test of HepG2 cells type by 2000X fluorescent microscope, HepG2 cells were treated with 100 μg.ml^{-1} of OGA (oligogalacturonides) or 2 μg.ml^{-1} of DDP (cisplatin) at 37°C for 24 h. Data expressed as mean ± SD with different superscript letters indicate statistical significance from five replications analyzed by one-way ANOVA with Duncan's post-hoc

Conclusions

In conclusion, oligogalacturonide (OGA) derived from citrus pectin exhibits synergistic suppressive effects on hepatocellular carcinoma (HCC) cells when combined with cisplatin (DDP), especially with DDP 12 h + OGA 12 h treatment. The OGA-DDP combination resulted in reduced cancer cell viability, increased lactate dehydrogenase (LDH) release, cell cycle arrest in the G2/M phase, elevated galectin-3 release, and decreased mitochondrial membrane permeability (MMP) compared to the effects of DDP or OGA alone. By enhancing cisplatin's anticancer effects, this combination could potentially reduce the required dosage of cisplatin, thereby minimizing its adverse side effects. These findings suggest that the OGA-DDP combination may offer a potential therapeutic approach for HCC. Future experiments are needed to study the impact of OGA-DDP *in vivo* and its long-term effects. Future studies should explore its pharmacokinetics, toxicity, and efficacy in preclinical animal models to assess its translational potential for clinical applications.

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Author Contributions

Conceptualization, C.S.H, D.L and M.C.W.; methodology, C.S.H, R.K.C and D.L.; formal analysis, C.S.H, R.K.C and D.L.; investigation, C.SH., R.K.C., P.H.W., and D.L..; resources, Y.T.W., and M.C.W.; data curation, C.S.H, R.K.C and D.L.; writing – original draft preparation, C.S.H, R.K.C and D.L; writing - C.SH., R.K.C., P.H.W., Y.T.W, M.C.W. and D.L., review and editing, R.K.C, Y.T.W., M.C.W. and D.L.; supervision, Y.T.W, D.L. and M.C.W.; All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

No animals were used in the present study. Therefore, separate permission was not required.

Informed Consent Statement

Not applicable.

Data Availability Statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest

The authors declare no conflicts of interest.

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